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A Novel Family of Genes Encoding Putative Pheromone Receptors in Mammals

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Summary

In mammals, olfactory sensory perception is mediated by two anatomically and functionally distinct sensory organs: the main olfactory epithelium (MOE) and the vomeronasal organ (VNO). Pheromones activate the VNO and elicit a characteristic array of innate reproductive and social behaviors, along with dramatic neuroendocrine responses. Differential screening of cDNA libraries constructed from single sensory neurons from the rat VNO has led to the isolation of a family of about 30 putative receptor genes. Sequence analysis indicates that these genes comprise a novel family of seven transmembrane domain proteins unrelated to the receptors expressed in the MOE. Moreover, the expression of each member of the gene family is restricted to a small subpopulation of VNO neurons. These genes are likely to encode mammalian pheromone receptors.

Introduction

Sensory systems receive information from the environment and transmit these signals to higher cortical centers in the brain, where they are processed to provide an internal representation of the external world. Mammals possess an olfactory system of enormous discriminatory power. Humans, for example, are capable of recognizing thousands of discrete odors. The perception of odors in humans is often viewed as an aesthetic sense, a sense capable of evoking emotion and memory, leading to measured thoughts and behaviors. Smell, however, is also the primal sense. In most species, odors can elicit innate and stereotyped behaviors that are likely to result from the nonconscious perception of odors. These different pathways of olfactory sensory processing are thought to be mediated by two anatomically and functionally distinct olfactory sensory organs, the main olfactory epithelium (MOE) and the vomeronasal organ (VNO) (Figure 1).

In mammals, the sensory epithelium of the main olfactory system resides within the posterior recess of the nasal cavity, whereas the VNO resides more anteriorly, in a blind-ended pouch within the septum of the nose (Jacobson, 1811; reviewed by Halpern, 1987; Wysocki, 1989; Farbman, 1992). The sensory neurons of both the MOE and VNO are bipolar. The dendrites terminate in specialized microvilli or cilia that bind odorants and transduce specific odorant binding into neural activity. The axons from sensory neurons of the MOE project through the skull to the main olfactory bulb, the first relay station in the brain. The main olfactory bulb then sends most of its fibers to the olfactory cortex, which in turn projects to higher sensory centers. The vomeronasal system, however, transmits olfactory information via a separate pathway of neuronal projections. The neurons of the VNO send axons to the accessory olfactory bulb, which projects to a discrete locus within the amygdala, distinct from the zone that receives fibers from the main olfactory pathway (Broadwell, 1975; Scalia and Winans, 1975; Winans and Scalia, 1970). The vomeronasal nucleus in the amygdala, in turn, sends fibers directly to the hypothalamus (Kevetter and Winans, 1981; Krettek and Price, 1977, 1978). Thus, the VNO pathway bypasses higher cognitive centers, resulting in innate and stereotyped behavioral and neuroendocrine responses.

What chemical signals activate the VNO, and what responses do they elicit? The VNO is largely responsive to olfactory cues secreted by other individuals within a species. These chemical signals provide information about gender, dominance, or reproductive status and elicit innate social and sexual behaviors, along with profound neuroendocrine changes (reviewed by Halpern, 1987; Wysocki, 1989; Wysocki and Lepri, 1991). In male rodents, for example, removal of the VNO in virgin animals severely impairs sexual responses, resulting in a dramatic reduction in the frequency of mating (Clancy et al., 1984; Meredith, 1986). In female rodents, activation of the VNO can induce puberty and estrus in the presence of males and prevent estrus in group-housed females (Lomas and Keverne, 1982; Johns et al., 1978; Reynolds and Keverne, 1979). Similarly, lesions in the vomeronasal system dramatically diminish male-specific aggressive behaviors (Bean, 1982; Clancy et al., 1984). The chemical signals responsible for eliciting these behaviors have been broadly defined as pheromones. Two classes of steroids, 16-androstenes and estrogens, can elicit reproductive behaviors in some mammals (Melrose et al., 1971; Michael and Keverne, 1968); F-prostaglandins and steroids elicit sperm production and mating in fish (Stacey and Sorensen, 1986; Sorensen et al., 1988), and small fatty acids, in association with the protein aphrodisin, have been implicated in the male sexual response in hamsters (Henzel et al., 1988; Singer, 1991). In most instances, however, the chemical nature of the odorants responsible for innate behavioral responses has not been elucidated.

Neither the pheromone receptors nor the signal transduction pathways activated by pheromones in VNO neurons have been identified. In the MOE, the repertoire of odorant receptor genes consists of about 1000 genes, each encoding a distinct seven transmembrane domain protein (Buck and Axel, 1991; Parmentier et al., 1992; Ben Arie et al., 1994). Analysis of the expression patterns of this family of odorant receptor genes (Ngai et al., 1993; Ressler et al., 1993, 1994; Vassar et al., 1993, 1994), coupled with earlier electrophysiologic and tracing experi-

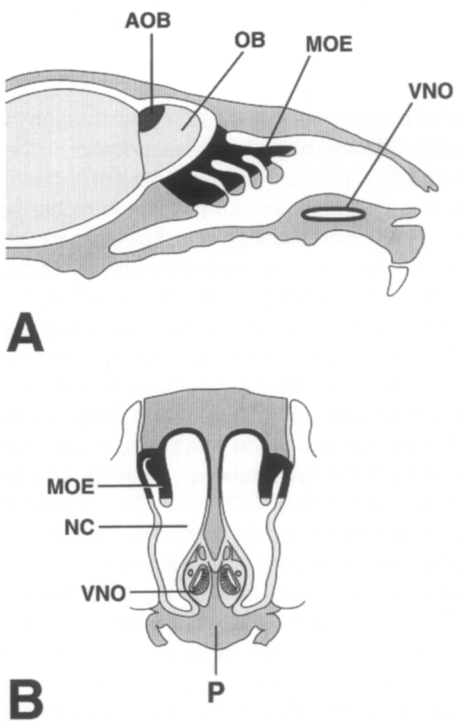

Figure 1. Spatial Segregation of the VNO and the Main Olfactory Systems

(A) A drawing of a parasagittal section through the skull of a rat. The convoluted turbinates of the main olfactory system (MOE) reside within the posterior recess of the nasal cavity (NC), whereas the VNO resides more anteriorly, in a blind-ended pouch within the septum of the nose. The axons from sensory neurons of the MOE project to the main olfactory bulb (OB), whereas the neurons of the VNO send axons to the anatomically distinct, more posteriorally placed accessory olfactory bulb (AOB). P, palate.

(B) A drawing of a coronal section showing the anatomically distinct VNO and the MOE. NC, nasal cavity; P, palate.

ments (Kauer et al., 1987; Stewart et al., 1979; Lancet et al., 1982; Mori et al., 1992; Imamura et al., 1992; Katoh et al., 1993), has provided a logic for olfactory discrimination. Individual sensory neurons in the MOE are likely to express only one of the 1000 receptor genes (Ngai et al., 1993; Chess et al., 1994; C. D. and R. A., unpublished data). Neurons expressing a given receptor, although randomly distributed in domains of the epithelium, project their axons to a small number of topographically fixed loci (or glomeruli) in the main olfactory bulb (Vassar et al., 1994; Ressler et al., 1994). These data support a model of olfactory coding in which discrimination of odor quality would result from the detection of specific spatial patterns of activity in the olfactory bulb.

The isolation of the genes encoding the pheromone receptors from VNO neurons might similarly provide insight into the chemical nature of the pheromones themselves, the logic of olfactory coding in the VNO, and the way in which perception of this class of odors leads to innate behaviors. Our efforts to identify the genes encoding the mammalian pheromone receptors by virtue of potential homology with the family of odorant receptor genes expressed in the MOE have been unsuccessful. We therefore developed a cloning strategy in which cDNA libraries were constructed from individual rat VNO neurons. Difference cloning permitted the identification of about 30 genes that define a novel family of presumed seven transmembrane domain receptors that are evolutionarily independent of the odorant receptors of the MOE. Expression of the individual members of this gene family is restricted to a distinct set of VNO neurons such that different neurons express different receptor genes. These genes are likely to encode mammalian pheromone receptors.

Results

Experimental Strategy

Our initial efforts to identify the genes encoding the pheromone receptors were based upon the assumption that the MOE and the VNO might share a common evolutionary origin such that DNA sequence homology may exist between the two receptor families. However, low stringency hybridizations of MOE receptor probes to rat VNO cDNA libraries, as well as polymerase chain reactions (PCRs) using conserved motifs from both the family of odorant receptor genes, as well as from the superfamily of known seven transmembrane domain receptors, were consistently unsuccessful. Moreover, the components of the olfactory signal transduction cascade in the MOE (the olfactory-specific G protein, G_{olf} [Jones and Reed, 1989]; the olfactory-specific adenylate cyclase [Bakalyar and Reed, 1990]; and the cyclic nucleotide responsive ion channel [Dhallan et al., 1990; Ludwig et al., 1990]) were not detectable in VNO neurons by in situ hybridization or by screening cDNA libraries (data not shown). These observations suggested that the pheromone receptors and the signal transduction pathways that they activate might have evolved independently in the VNO and the MOE.

We therefore developed a cloning procedure that made no assumptions concerning the structural class of the receptor molecules. Rather, we only assumed that the expression of the pheromone receptors would be restricted to the VNO and that individual neurons within the VNO were likely to express different receptor genes. In the MOE, about 1% of the mRNA in a given sensory cell encodes a given receptor (Vassar et al., 1994). However, the 1000 different receptor genes are each expressed in different neurons such that the frequency of a specific receptor RNA will be diluted to 0.001% of the mRNA message population. The generation of libraries from individual neurons provided an experimental solution to the problem of detecting a specific mRNA in a heterogeneous population of neurons. Reverse transcription PCR was therefore used to generate double-stranded cDNA, as well as cDNA libraries from individual VNO sensory neurons. We expected that the frequency of a specific receptor cDNA in libraries from single neurons would be about 1%. Differential screening of such libraries from single neurons should therefore permit the isolation of pheromone receptor genes.

In control experiments, the cDNA library prepared from a single rat VNO neuron was screened with probes for tubulin and olfactory marker protein (OMP) to determine

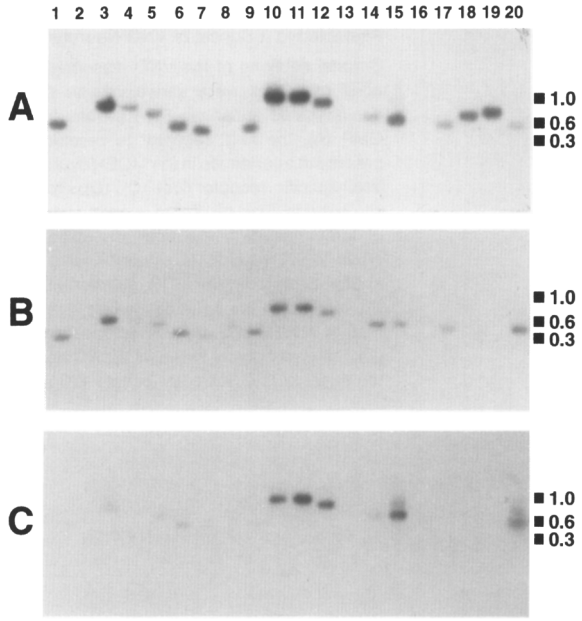

Figure 2. Identification of cDNA Clones Specifically Expressed in an Individual VNO Neuron

We isolated 20 cDNA clones initially identified by differential screening of a cDNA library from a single VNO neuron. The inserts were amplified by PCR, electrophoresed on 1% agarose gels, and blotted to nylon filters. Blots were annealed with ^{32}P-labeled cDNA probe from *VN1* (A), *VN2* (B), or a neuron from the MOE (C). Two cDNA clones (18 and 19) only anneal with cDNA prepared from *VN1*. One clone (17) anneals with the cDNA from both VNO neurons, but not with cDNA from an MOE neuron.

whether these libraries accurately represent the mRNA population. The frequency of these clones suggested that the representation of a given RNA was not biased in the construction of the library (see Experimental Procedures). We then screened in triplicate 1000 recombinant phages from a cDNA library prepared using probes from VNO neuron 1 (*VN1*), a second VNO neuron (*VN2*), and a neuron from the MOE. About 2% of the cDNA clones screened showed specific hybridization with cDNA probes from *VN1*, but not with probes from *VN2* or the MOE neuron. The specificity of these cDNA clones was further examined in a more sensitive assay. The inserts from these cDNA clones were amplified by PCR, and the DNA products were hybridized on Southern blots with cDNA probes from *VN1*, *VN2*, or an MOE sensory neuron (Figure 2). Of 20 clones initially isolated from the *VN1* cDNA library, only two (clones 18 and 19 in Figure 2A) appeared to be specific to *VN1* in this more sensitive screen. These two clones represented independent isolates of an identical cDNA sequence present within the cDNA library of *VN1* at a frequency of 0.5%. This cDNA was used as a probe to isolate full-length clones from a cDNA library with larger inserts constructed with RNA prepared from several dissected VNOs. A full-length clone, *VN1*, encodes a seven transmembrane domain receptor (see below).

The pattern of expression of this cDNA was determined by performing RNA in situ hybridization to sections through the rat VNO. In cross section, a thick multicellular sensory epithelium lines half of the lumen of the VNO (Figure 3). In situ hybridization demonstrates that mature VNO neurons uniformly express OMP (Figure 3A). In contrast, the cDNA specific for *VN1* localized to a subpopulation of VNO neurons (Figure 3C). No hybridization was observed in the MOE (Figure 3D) or in any other neural or nonneural cells (see below).

Thus, difference cloning from libraries prepared from single neurons has allowed the isolation of a novel seven transmembrane domain receptor expressed in VNO sensory neurons.

The Sequence of Several Members of the Receptor Gene Family

We observed that *VN1* is expressed in about 4% of the VNO sensory neurons. This suggested the existence of a gene family with individual member genes expressed in different subsets of neurons. We therefore used both PCR and high and low stringency hybridization to VNO cDNA libraries to identify possible members of a receptor gene family expressed in other VNO neurons (see Experimental Procedures). The sequences of seven different cDNAs obtained in this manner are aligned in Figure 4. Hydropathy analysis suggests that each of the seven sequences contain seven hydrophobic stretches that represent potential transmembrane domains. Sequence analysis suggests that these putative receptors are likely to adopt a structure similar to that of the previously characterized superfamily of seven transmembrane receptors. However, the VNO receptors do not share any of the conserved sequence motifs exhibited by members of the previously identified superfamily (Baldwin, 1993; Probst et al., 1992). One region of homology, however, is observed with the family of mammalian prostaglandin receptors throughout the second and third transmembrane domains (Figure 4B). We observed 25% identity between *VN2* and the rat E3 prostaglandin receptor over these two domains, but no significant sequence homology is observed in other regions of the molecule. Prostaglandins are potent pheromones eliciting mating in fish, but their role as mammalian pheromones is unknown. However, this level of homology over a small region of the protein does not permit us to argue that the receptors may recognize prostaglandins.

Overall, the seven VNO cDNA sequences share between 47% and 87% sequence identity. As observed previously for the odorant receptors from the MOE (Buck and Axel, 1991), this family of VNO receptors exhibits significant divergence within the transmembrane domains, the presumed site of ligand binding (Strader et al., 1994). This pattern of divergence suggests that the different members may permit the binding of different structural classes of ligands.

The Size of the Gene Family

We have analyzed the size of the vomeronasal receptor gene family by performing hybridizations to genomic DNA, as well as quantitative screening of genomic libraries. The seven cDNAs that we have characterized fall within six subfamilies as defined by the observation that no cross-hybridization is observed among the different subfamilies

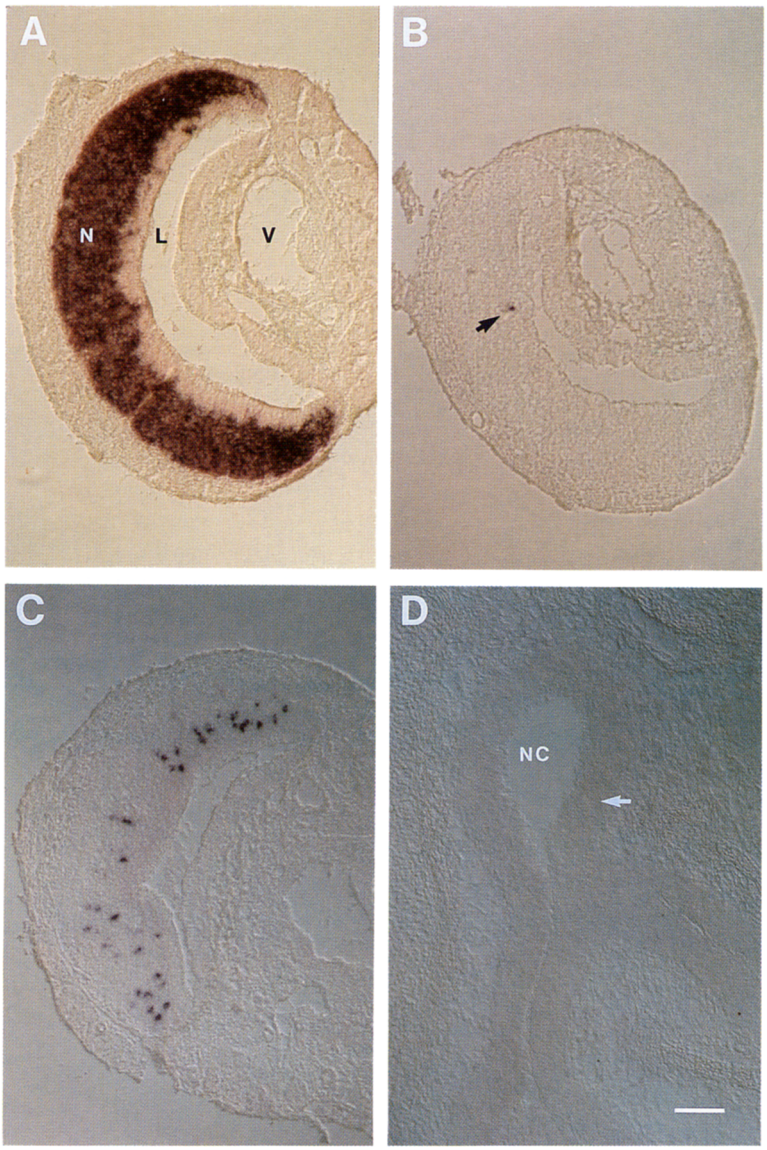

Figure 3. Expression of *VN1* Receptor RNA Is Restricted to a Subset of VNO Neurons

Coronal sections of the VNO dissected from adult male rats were annealed with digoxigenin-labeled antisense RNA probes for the OMP (A), the M12 receptor (a receptor expressed in abundance in the MOE) (B), and the VNO-specific receptor *VN1* (C). (D) shows in situ hybridization of *VN1* to a coronal section of turbinates from the newborn MOE. The arrow in (B) indicates a single positive VNO neuron expressing the MOE receptor, M12. In (A), N denotes the neuroepithelium; L, the lumen of VNO; and V, the vomeronasal vein. In (D), the arrow points to the MOE; NC denotes the nasal cavity. Scale bar equals 120 μm.

under high stringency conditions. cDNA probes from each of the six subfamilies were then annealed to Southern blots of rat genomic DNA after digestion with two different restriction endonucleases (Figure 5). The VNO receptor genes analyzed thus far do not contain introns within the coding region (data not shown). Restriction cleavage was performed with endonucleases that do not cleave within the cDNAs we have isolated such that the number of hybridizing bands will closely approximate the number of receptor genes. Probes from each of the subfamilies identified from two to eight bands in genomic DNA such that a total of about 20 bands were detected in hybridizations with the six individual probes. A mix of six probes identifies about 20 bands in genomic DNA at high stringency of hybridization (Figure 5H) and more than 30 bands under less stringent conditions (Figure 5I).

An independent estimate of the size of the gene family was obtained by screening a genomic library. A mix of the seven cDNA clones was used as a hybridization probe under reduced stringency conditions to identify about 35 positive clones per haploid genome. Thus, the data from Southern blotting and screens of genomic library are in accord with one another and indicate that the multigene family of VNO receptors we have identified consists of between 30 and 40 genes.

The Pattern of Receptor Expression in the VNO

We performed in situ hybridization to examine the spatial pattern of receptor expression in the sensory epithelium of the VNO. The VNO consists of a blind-ended tubular structure that extends in an anterior–posterior dimension within the septum. In cross section, the sensory epithelium lines the medial half of the tube and a vein surrounded by nonneuronal tissue resides more laterally (see Figure 3A; Figure 6). RNA in situ hybridization experiments were performed with digoxigenin-labeled RNA antisense probes

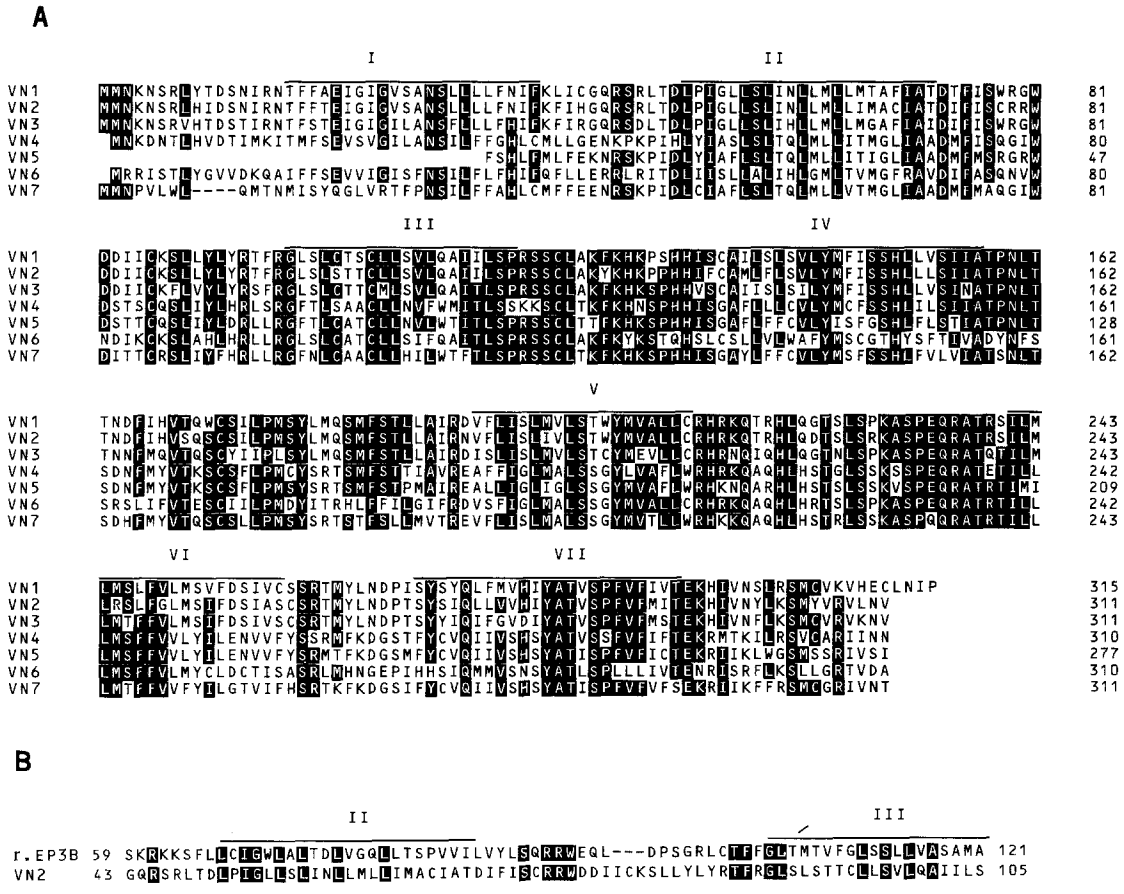

Figure 4. Deduced Amino Acid Sequences of the Pheromone Receptor cDNAs

(A) The deduced amino acid sequences of seven putative pheromone receptor cDNAs are aligned. Predicted positions of the seven transmembrane domains are indicated (I–VII). Amino acid residues common to at least five of the seven sequences are shown as white lettering on black background. (B) An alignment between the sequences of the second and third transmembrane domains of the rat prostaglandin receptor E3 (rEP3B) and the VNO receptor *VN2* showing 28% identity over this region of the receptor sequence.

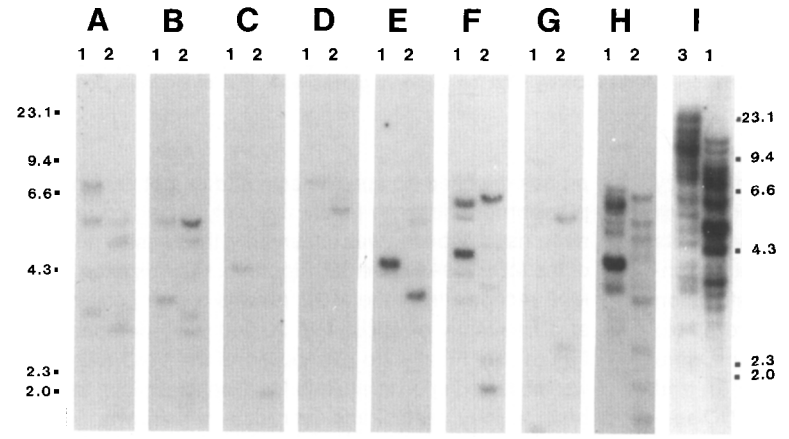

Figure 5. Southern Blot Analysis with the Seven Pheromone Receptor cDNAs

Rat genomic DNA isolated from liver was digested with PstI (lanes 1) or EcoRI (lanes 2), electrophoresed on 0.8% agarose gels, and blotted to nylon filters. Blots were annealed with ^{32}P-labeled probes corresponding to the seven different receptor cDNAs, *VN1* to *VN7* (shown in [A]–[G], respectively). Under the high stringency conditions of hybridization and washing used in these experiments, cross-hybridization is observed between *VN1* and *VN2*, whereas the other individual receptor probes do not cross-hybridize. A mix of six probes specific for each of the six receptor subfamilies (*VN2* to *VN7*) was annealed under conditions of high (H) and lower (I) stringency to either PstI-cleaved (lanes 1), EcoRI-cleaved (lanes 2), or HindIII-cleaved (lane 3) DNA (see Experimental Procedures). (I) was run separately from (A)–(H), which were electrophoresed on the same gel.

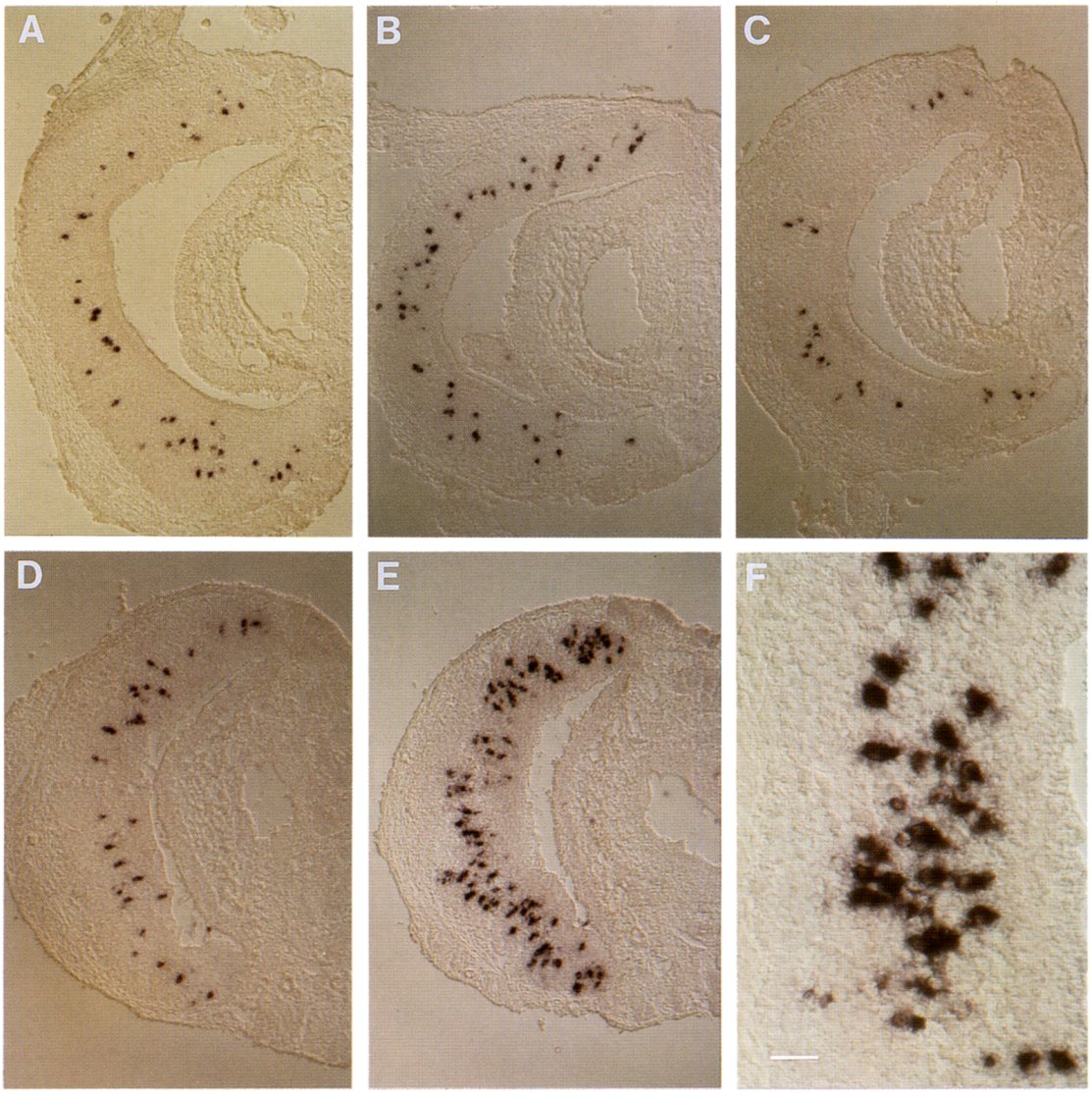

Figure 6. Localization of the Individual Receptors to Distinct Subpopulations of Cells within the VNO

In situ hybridization to coronal sections of a dissected VNO using digoxigenin-labeled probes from either the individual receptors or a mix of the six receptors. Digoxigenin-labeled antisense RNA probes from receptor *VN1* (A), receptor *VN3* (C), receptor *VN4* (D), or a mix of six probes specific for each receptor subfamilies (E) were annealed to a coronal section of the VNO dissected from male rats. (B) shows the annealing of receptor *VN1* probe to a section through the VNO from a female rat. (F) shows a high power magnification of (E). VNO cDNA clones 1–7 label 2.7%, 3.8%, 1.1%, 1.2%, 1.1%, 1.5%, and 3% of the cells in the neuroepithelium, respectively. The mix of seven probes label 15% of the cells. Scale bar equals 120 μm.

from each of the six subfamilies under high stringency conditions, such that it was likely that a given probe will only detect members within its own subfamily. The results with each of the six probes were qualitatively indistinguishable. In each case, we observed a punctate distribution of cells expressing a given receptor RNA (Figure 6). No differences in the patterns of in situ hybridization were observed between males and females (Figures 6A and 6B). Each probe detected about 1%–4% of the VNO sensory neurons. These data contrast with hybridization patterns observed with the probe for OMP (see Figure 3A), which demonstrated uniform labeling of the VNO epithelium. Control sections hybridized with sense receptor probes revealed no specific signal (data not shown). Expression of this gene family was only observed in VNO neurons; no labeling was observed in the sensory neurons of the MOE (see Figure 3D; Figure 7). Hybridization of the M12 receptor from the MOE reveals a rare positive cell at a frequency of about 1 in 20,000 VNO neurons (see Figure 3B). Finally, no expression of the VNO receptors was observed upon in situ hybridization to sections through brain, kidney, testes, and liver (data not shown).

Analysis of several sections through the entire VNO suggested that neurons expressing a given receptor are not topologically localized but rather are randomly distributed along the anterior–posterior axis. In cross section, how-

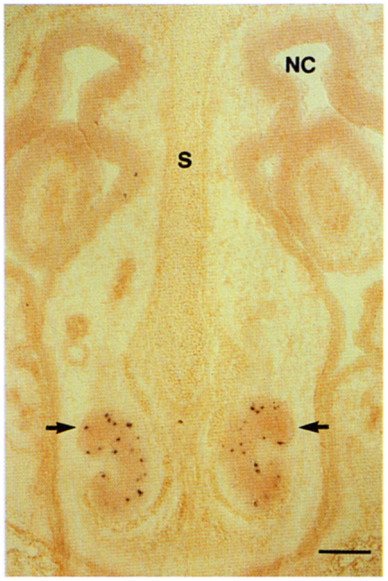

Figure 7. Receptor Expression Is Restricted to VNO Neurons

A coronal section through the head of an E17 rat shows hybridization of a mix of six receptor probes to neurons within the VNO (arrows), but not to neurons within the MOE nor to other tissues in the nose. NC, nasal cavity; S, septum. Scale bar equals 250 μm.

ever, neurons expressing the receptor family we have cloned are preferentially localized to the apical two thirds of the zone of OMP-positive cells. Previous studies in the opossum have demonstrated that this apical zone of neurons expresses the G protein $G_{i\alpha 2}$, whereas the more basal zone expresses G_o (Halpern et al., 1995), and a similar pattern is observed in rat (L. Belluscio, C. D., and R. A., unpublished data). Therefore, the expression of the family of receptors we have isolated may be restricted to $G_{i\alpha 2}$-positive cells. It is possible that the G_o-positive cells express a more distant family of receptors.

Individual Neurons Express Different Complements of Receptors

In the MOE, a given neuron is likely to express only one receptor from the family of 1000 receptor genes. Moreover, neurons expressing a given receptor project their axons to one or a small number of topographically defined glomeruli within the olfactory bulb. The regulated expression of odorant receptors assuring that only one receptor is expressed in individual olfactory neurons is an important element in the coding of olfactory information in the main olfactory system. Quantitative analysis of the in situ hybridizations of the VNO receptor probes indicates that neurons within the VNO similarly express only a single receptor gene.

The observation that 1%–4% of the VNO neurons express a given receptor subfamily suggests that each cell expresses only a subset of receptor genes. If we demonstrate that each of the different receptor probes hybridizes with distinct nonoverlapping subpopulations of neurons, this would provide evidence that neurons differ with respect to the receptors they express. Sections were annealed with probes specific for each of the six receptor subfamilies or with a mixture of six probes (see Figure 6). If each receptor is expressed in a distinct nonoverlapping subpopulation of neurons, then the sum of the cells identified with the six probes should equal the number of cells identified with the mixed probe. In accord with this suggestion, we observed that the percentage of olfactory neurons detected with the mixed probe (15%) is significantly greater than the percentage detected with any of the individual probes alone and approximates the sum of the percentage of positive neurons detected with the six individual probes (12%). These values are presented in the legend to Figure 6. These results suggest that the six receptor subfamilies are expressed in distinct nonoverlapping populations of olfactory neurons and provide support for a model in which a single sensory neuron expresses a single receptor gene.

Discussion

We have identified a novel family of seven transmembrane domain proteins that is likely to encode the mammalian pheromone receptors. Differential screening of cDNA libraries constructed from single sensory neurons initially led to the isolation of a family of putative receptor genes. Each member of the gene family is expressed in a small subpopulation of neurons such that the seven putative receptor genes we have cloned identify 15% of the cells in the VNO. The expression of this gene family is restricted to neurons within the VNO and is not observed in sensory neurons of the MOE nor in other nonneuronal cells. This array of properties is consistent with those predicted for the mammalian pheromone receptors. Proof that these sequences indeed encode pheromone receptors will require the demonstration that these receptor proteins bind pheromones and are able to transduce pheromone binding into alterations in membrane potential.

Three large gene families have now been identified whose members are expressed in subsets of olfactory neurons in the nematode Caenorhabditis elegans (Troemel et al., 1995 [this issue of *Cell*]) and in the MOE (Buck and Axel, 1991) and VNO of vertebrates. What functions could we ascribe to these gene families if they do not encode odorant receptors? In the MOE, neurons expressing a given olfactory receptor project their axons to one or a small number of glomeruli within the olfactory bulb (Vassar et al., 1994; Ressler et al., 1994). It is formally possible, therefore, that these receptors recognize guidance cues that are spatially distributed within the olfactory bulb. An even more parsimonious model would argue that these receptors may recognize odorants in one pole of the cell, the dendrite, and recognize guidance molecules at the axon termini.

The experimental approach employed to isolate this gene family, differential screening of a cDNA library constructed from a single neuron, may be more broadly applicable to the analysis of the specific gene expression in diverse populations of cells. In the nervous system, for

example, functionally distinct neurons each expressing different genes and each projecting to different targets are often interspersed. It has therefore been difficult to isolate RNA species unique to functionally distinct subsets of neurons within a heterogeneous cell population. The ability to generate cDNA libraries from individual cells within a diverse population of neurons may permit the identification of that subset of genes that afford a cell a unique identity.

How Large Is the Gene Family?

The number of receptor genes expressed in the two distinct olfactory organs of mammals is likely to reflect the repertoire of odors recognized by the two populations of olfactory sensory neurons. The main olfactory organ can recognize a universe of odors that define the environment of an organism, whereas the VNO largely recognizes molecules distinctive to the species that define the reproductive and social status of individuals within any given species. Olfactory receptors of the MOE are encoded by a family of about 1000 genes (Buck and Axel, 1991; Parmentier et al., 1992; Ben Arie et al., 1994). Since the range of molecules detected by the VNO is thought to be far smaller than the odors detected by the MOE, we anticipated that the repertoire of pheromone receptors would be far smaller as well. Gene cloning and Southern blotting with genomic DNA provide an estimate of the size of the pheromone receptor repertoire. A screen of genomic libraries with a mix of probes detect approximately 35 positive clones per genome. This value is in accord with the results of genomic blot hybridization at low stringency that identifies about 30 discrete genes with the available probes. This estimate of a minimum of 30–35 genes clearly provides a lower limit of the size of the VNO receptor repertoire since it is likely that the seven genes we have cloned do not allow us to detect all the members of the pheromone receptor gene family.

In situ hybridization experiments with individual probes provide an independent estimate of the number of receptor genes expressed in the VNO. Each of the seven putative pheromone receptor genes labels about 1%–4% of the VNO sensory neurons, whereas a mix of the genes representing the six subfamilies detects about 15% of the VNO neurons. These data suggest that a given neuron expresses only one pheromone receptor gene. Since the six subfamily probes detect about 20 genes in the chromosome at high stringency and label 15% of the VNO neurons, we estimate that the repertoire of pheromone receptors may consist of about 100 distinct genes.

The Relationship between the Two Olfactory Organs

The sequences of the odorant receptors of the MOE and the pheromone receptors of the VNO share no apparent homology, indicating that the two olfactory sensory systems of mammals have evolved independently. This suggestion is in accord with the observation that the signal transduction machinery of the MOE cannot be detected in the neurons of the VNO. What is the evolutionary origin of the VNO? Pheromone-responsive neurons and neurons responsive to the more general class of odorants are likely to have been present throughout vertebrate evolution. With the emergence of terrestrial forms, segregation of the two types of neurons may have occurred, generating a distinct VNO that facilitates the access and binding of the two classes of odorous ligand. Thus, terrestrial vertebrates from amphibians to mammals, including humans, retain two distinct olfactory systems, the VNO and the MOE (Bertmar, 1981; Eisthen, 1992; Potiquet, 1891; Stensaas et al., 1991; Moran et al., 1991; Garcia-Velasco and Mondragon, 1991).

These two functional classes of sensory neurons are also apparent in invertebrate olfactory systems. These observations immediately pose the question as to whether homologs of the two different families of vertebrate olfactory receptors are present within the genome of invertebrates. Attempts to identify genes related to the large family of MOE receptors in C. elegans (C. Bargmann, personal communication) and Drosophila (H. Amrein, L. Vosshall, and R. A., unpublished data; J. Carlson, personal communication) have thus far been unsuccessful. Several large families of seven transmembrane receptor genes expressed in subsets of C. elegans chemosensory neurons have recently been identified (Troemel et al., 1995). However, these sequences share no homology with the mammalian receptor sequences from either the VNO or MOE. It is possible that the identification of additional families of receptors will reveal a common evolutionary ancestor to the vertebrate and invertebrate olfactory systems. Alternatively, the differences in the chemical nature of the odorants and differences in the physiological consequences of odor recognition might suggest independent origins for the invertebrate and vertebrate olfactory system.

The Logic of Olfactory Coding in the MOE and VNO

Analysis of the patterns of expression of receptor genes in the main olfactory system has provided significant insight into mechanisms for the diversity and specificity of odor recognition in mammals. Similarly, the isolation of the pheromone receptors from the VNO is likely to help to elucidate the logic of olfactory perception in the vomeronasal system. The initial step in olfactory discrimination by the MOE requires the interaction of odorous ligands with one of the multiple seven transmembrane domain receptors on olfactory sensory neurons. Discrimination among odorants requires that the brain determine which of numerous receptors has been activated. Since individual olfactory sensory neurons in the MOE are likely to express only a single receptor gene, the problem of distinguishing which receptors have been activated reduces to a problem of distinguishing which neurons have been activated.

Recent experiments demonstrate that neurons expressing a given receptor, and therefore responsive to a given odorant, project their axons to one or a small number of discrete loci or glomeruli within the olfactory bulb (Vassar et al., 1994; Ressler et al., 1994; P. Mombaerts et al., personal communication). The positions of specific glomeruli are topographically fixed and are conserved in the

brains of all animals within a species. These data provide physical evidence for a two-dimensional map within the olfactory bulb that identifies which of the numerous receptors have been activated within the sensory epithelium. Such a model is in accord with previous experiments demonstrating that different odors elicit spatially defined patterns of glomerular activity in the olfactory bulb (Kauer et al., 1987; Stewart et al., 1979; Lancet et al., 1982; Mori et al., 1992; Imamura et al., 1992; Katoh et al., 1993). Thus, the quality of an olfactory stimulus would therefore be encoded by the specific combination of glomeruli activated by a given odorant.

At one level, the vomeronasal system shares anatomic and physiologic features with the main olfactory system, suggesting that similar experiments with pheromone receptors might also provide insight as to how the recognition of odors by the VNO leads to the elaboration of innate behaviors. Primary olfactory sensory neurons within the VNO project a single unbranched axon that then synapses with dendrites of mitral cells in the accessory olfactory bulb, the first relay station for vomeronasal signaling in the brain. At a molecular level, we have identified a family of putative pheromone receptor genes that encode seven transmembrane domain proteins. Individual VNO neurons are likely to express only a single receptor gene. Cells expressing a specific receptor are randomly dispersed within the apical zone of the sensory epithelium. Thus, the pattern of pheromone receptor expression shares striking similarities with the expression of odorant receptors in the MOE.

At first glance, the anatomy and molecular organization of the VNO and MOE as well as that of the main and accessory olfactory bulb appears quite similar. There are, however, important differences. In the MOE, the mitral cells, the major output neurons of the olfactory bulb, project a primary dendrite to a single glomerulus, suggesting a one-to-one correspondence between mitral cell and sensory axon, such that a given mitral cell can respond to the activation of only a single class of sensory neurons. The task of discerning which sensory neurons have been activated must therefore be accomplished by integration at higher cortical centers. Mitral cells of the accessory bulb, however, exhibit a more complex primary dendritic array, allowing synapse formation with more than one glomerulus and therefore more than one class of sensory neurons (Macrides et al., 1985; Takami and Graziadei, 1991). These observations suggest that in the vomeronasal system, integration permitting the detection of a specific combination of different receptors activated by pheromones may occur in the accessory olfactory bulb.

The VNO and the main olfactory system reveal striking differences in the secondary projections to the cortex and in the responses elicited by the two sensory systems. VNO neurons project directly to the amygdala and hypothalamus, leading to innate and stereotypic behavioral responses (Broadwell, 1975; Scalia and Winans, 1975; Winans and Scalia, 1970; Kevetter and Winans, 1981; Krettek and Price, 1977, 1978). In contrast, the projections from the main olfactory organ activate higher cortical centers, resulting in a measured emotional or cognitive response. The projections from the vomeronasal system to the hypothalamus also control the release of luteinizing hormone release hormone and prolactin release hormone, increasing luteinizing hormone and prolactin levels both centrally and peripherally (reviewed by Keverne, 1983; Meredith and Fernandez-Fewell, 1994). In this manner, stimulation of the vomeronasal system can coordinate the activation of central neural pathways with dramatic neuroendocrine changes to elicit a characteristic array of innate reproductive and social behaviors.

The coding of olfactory information is likely to be far simpler in the vomeronasal system than in the main olfactory pathway. The receptor repertoire in the VNO is an order of magnitude smaller than in the MOE. Moreover, integration in the vomeronasal pathway is apparent in the accessory bulb and the secondary projections synapse on small number of loci in the amygdala. This is in sharp contrast with the complexity of higher cortical pathways required for processing olfactory information from the MOE. Thus, the vomeronasal system may permit the analysis of the molecular events that translate the bindings of pheromones into innate stereotypic behaviors.

Pheromone Receptors in Humans

Until recently, the VNO in humans was thought to be an atretic organ of vestigial function. Recent reports, however, identify a structurally intact VNO in virtually all biopsy specimens examined (Moran et al., 1991; Stensaas et al., 1991; Garcia-Velasco and Mondragon, 1991). Activation of neurons has been observed in the human VNO in response to purified components from skin extracts (Monti-Bloch et al., 1994), but the physiological or behavioral consequences of VNO activation remain elusive. Moreover, it has been difficult to identify human pheromones that elicit innate behavioral arrays since behavior in humans is far more likely to be tempered by learning and experience.

In preliminary experiments, we have identified homologs of the rodent VNO receptors in human genomic DNA. Low stringency screens of a human genomic library with a mix of rat VNO receptor cDNAs identifies human homologs at a frequency of about 15 per haploid genome. Partial sequence of two clones reveals 41% and 48% identity with the closest rat homologs. However, both genomic clones reveal stop codons within the coding region, indicating that these two human sequences are pseudogenes. Characterization of additional genomic or cDNA clones from the human VNO will be required to determine whether humans indeed express functional VNO receptors. The identification of putative pheromone receptors may provide insight into the chemical nature of the pheromones, the mechanisms by which the perception of pheromones lead to innate behaviors and the possible role of this sensory system in humans.

Experimental Procedures

Preparation and Screening of Single-Cell cDNA Libraries

The MOE and VNOs were dissected from adult Sprague–Dawley rats. The synthesis and amplification of single-cell cDNA were performed

according to Brady et al. (1990) with modifications. Small pieces of tissue were dissociated for 10 min at 37°C in phosphate-buffered saline (PBS) (without Ca^{2+} or Mg^{2+}), 0.025% trypsin, 0.75 mM EDTA. After gentle trituration of the tissues in Dulbecco's modified Eagle's medium plus 10% calf serum, cells were collected by centrifugation and resuspended in ice-cold PBS. The cell suspension was observed on a Leitz inverted microscope, and olfactory sensory neurons were identified as bipolar neurons with an axonal process and a dendrite terminating in an olfactory knob. Isolated neurons were picked with a Leitz micromanipulator fitted with a pulled and beveled microcapillary. Single cells were seeded in thin-walled PCR tubes (Perkin–Elmer) containing 4 μl of ice-cold cell lysis buffer (50 mM Tris–HCl [pH 8.3], 75 mM KCl, 3 mM $MgCl_2$, 0.5% NP-40, containing 80 ng/ml pd(T)19-24 [Pharmacia], 5 U/ml prime RNase inhibitor [5′–3′ Incorporated], 324 U/ml RNAguard [Pharmacia], and 10 μM each of dATP, dCTP, dGTP, and dTTP). Lysis was subsequently performed for 1 min at 65°C. First-strand cDNA synthesis was then initiated by adding 50 U of Moloney murine leukemia virus and 0.5 U of avian reverse transcriptases (Bethesda Research Laboratories) followed by incubation for 10 min at 37°C. Samples were heat inactivated for 10 min at 65°C, and poly(A) was added to the first-strand cDNA product by adding an equal volume of 200 mM potassium cacodylate (pH 7.2), 4 mm $CoCl_2$, 0.4 mM DTT, 200 μM dATP containing 10 U of terminal transferase (Boehringer) for 15 min at 37°C. Samples were heat inactivated for 10 min at 65°C, and the contents of each tube was brought to 100 μl with a solution made of 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 100 μg/ml bovine serum albumin, 0.05% Triton X-100 and containing 1 mM of dATP, dCTP, dGTP, dTTP, 10 U of Taq polymerase (Perkin–Elmer), and 5 μg of the PCR primer *AL1*. The *AL1* sequence is ATT GGA TCC AGG CCG CTC TGG ACA AAA TAT GAA TTC $(T)_{24}$. PCR amplification was then performed according to the following schedule: 94°C for 1 min, 42°C for 2 min, and 72°C for 6 min with 10 s extension per cycle for 25 cycles. We then added five additional units of Taq polymerase before performing 25 more cycles. In this manner, PCR-amplified cDNA was synthesized from RNA of individual neurons.

Aliquots of single-cell cDNA were run on 1% agarose gels, blotted on nylon membrane (Hybond N^+, Amersham), and hybridized with several DNA probes to determine the representation of specific sequences in amplified cDNA. The probes included highly expressed genes (tubulin and OMP), a gene expressed at lower level (G_o), as well as genes whose expression is restricted to either MOE (G_{olf}) or VNO ($G_{i\alpha 2}$) neuron. The relative level of these genes in amplified cDNA prepared from individual neurons is in accord with levels determined by either in situ hybridization or screening more classical cDNA libraries. These data suggest that the amplified cDNA from individual neurons contains an accurate representation of sequences in mRNA.

Of the cDNA prepared from *VN1*, 1 μg was purified by phenol/chloroform extraction, digested with EcoRI, ligated into λZAPII phage arms predigested by EcoRI and dephosphorylated (Stratagene), and packaged according to standard procedures. The library prepared from *VN1* consisted of 5×10^4 pfu with an average insert size of 600 bp. The frequency of OMP- and tubulin-positive plaques (0.2%) suggested that the representation of a given RNA was not biased during the construction of the library.

Amplified cDNA from single cells was used as probe by reamplifying 1 μl of neuron cDNA for 10 cycles with the *AL1* primer in the presence of 100 μCi of [^{32}P]dCTP. We plated 1000 recombinant phages from *VN1* library at low density, and triplicate filters (Hybond N^+, Amersham) were prehybridized at 65°C in 0.5 M sodium phosphate buffer (pH 7.3) containing 1% bovine serum albumin and 4% SDS. Hybridization was carried out in the same buffer and at 65°C after adding 10^7 cpm/ml of the amplified cDNA probe made from either *VN1*, *VN2*, or an MOE neuron. Filters were washed three times at 65°C in 0.5% SDS and 0.5 × SSC. We isolated 20 phage plaques showing specific hybridization with the *VN1* probe. Phage inserts were amplified by PCR, run on 1% agarose gels, transferred to nylon membranes, and again hybridized with single-cell cDNA probes as described above. Phages 18 and 19 contained cDNA inserts that appeared to hybridize only to *VN1* cDNA probe. Plasmids were obtained from the isolated phages by performing phagemid rescue as instructed by the manufacturer (Stratagene). DNA sequence analysis was performed on plasmid DNAs using the Sequenase system (United States Biochemical Corporation).

Isolation and Analysis of Full-Length cDNA Clones

Poly(A)$^+$ RNA was isolated from VNOs dissected from adult male or female rats using the poly(A)$^+$ isolation kit (Stratagene) according to the instructions of the manufacturer. cDNA libraries were prepared in the λZAPII vector (Stratagene) according to standard procedures (Sambrook et al., 1989). Independent recombinant phages (2×10^6) from the male and female VNO cDNA libraries were screened under high stringency hybridization (68°C in 0.5 M sodium phosphate buffer [pH 7.3] containing 1% bovine serum albumin and 4% SDS) with a ^{32}P-labeled probe (Prime-It, Stratagene) prepared from the *VN1*-specific clone 18. This allowed the isolation of two full-length cDNA clones, *VN1* and *VN2*. In further screens, one additional cross-hybridizing cDNA clone, *VN3*, was obtained by low stringency hybridization (55°C in the same buffer as described above) of a mix of *VN1* and *VN2* probes to the VNO cDNA libraries. Conserved motifs within these cDNA clones were used to generate PCR primers that were then used to amplify additional sequences from the VNO cDNA libraries. This PCR product, along with the three cDNA clones, was used as probe in further hybridizations to obtain four additional full-length cDNAs.

Southern Blotting and In Situ Hybridization Analysis

Genomic DNA prepared from Sprague–Dawley rat liver was digested with the restriction enzyme EcoRI, PstI, or HindIII, size fractionated on 0.8% agarose gels, and blotted into nylon membrane (Sambrook et al., 1989). The membranes were cross-linked under UV light, prehybridized, and hybridized in 0.5 M sodium phosphate buffer (pH 7.3) containing 1% bovine serum albumin and 4% SDS at either high (68°C) or low (55°C) stringency conditions. λFIXII genomic libraries made from human placenta (Stratagene) and Sprague–Dawley rats were screened under low stringency conditions.

In situ hybridization was performed as described previously (Schaeren-Wiemers and Gerfin-Moser, 1993) using full-length clones *VN1* to *VN7* as templates to synthesize digoxygenin-labeled cRNA probes. Sequences corresponding to the BamHI–Asp718 fragment of OMP cDNA (Rogers et al., 1987) were used to synthesize a 1 kb OMP probe. The sequence encompassing the transmembrane domains 3 through 7 of MOE receptor M12 was isolated by PCR.

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